A monoclonal antibody against *Echinococcus multilocularis* Em2 antigen

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SUMMARY

A monoclonal antibody (MAb G11) species-specific to the Em2 antigen of *Echinococcus multilocularis* was generated for (i) further biological characterization of the Em2 antigen, (ii) easy affinity-purification of Em2 antigen for immunodiagnostic and immunological investigations and (iii) development of a sandwich-ELISA for the detection of Em2 antigen in diagnostic samples and thus species-specific identification of *E. multilocularis* metacestode material. The MAb G11 was used in an antibody sandwich-ELISA to detect soluble Em2 antigen with a methodical sensitivity of 80 ng *E. multilocularis* antigen/ml of solution. MAb G11 specifically detected Em2 antigen in all of 15 *E. multilocularis*-isolates originating from various geographical areas and in none of other helminth isolates (e.g. *Echinococcus granulosus*, *E. vogeli*, and others). Further biological analysis by FITC-labelled MAb G11 demonstrated unique binding activity to the laminated layer of the metacestode. Also, oncospheres were binding FITC-labelled MAb G11 on an outer layer synthesized during cultivation *in vitro* for 13 days after hatching. Application of the MAb G11 antibody sandwich-ELISA for investigation of solubilized oncospheres confirmed the *in vitro* synthesis of Em2 antigen by oncospheres on day 13 p.i. Adult stages (somatic antigens) and freshly hatched oncospheres were always MAb G11 negative. Solid-phase MAb G11 was used for purification of the corresponding Em2 antigen by affinity chromatography. A preliminary serological evaluation of the Em2(G11) antigen by ELISA revealed identical immunodiagnostic characteristics, compared to Em2 obtained by classical means, thus suggesting the presented method for future isolation of large-scale Em2 antigen.

Key words: *Echinococcus multilocularis*, Em2 antigen, monoclonal antibody, affinity chromatography, Em2-sandwich-ELISA.

INTRODUCTION

Alveolar echinococcosis of humans is caused by infection with the metacestode (larval stage) of *Echinococcus multilocularis*. Early serological detection and treatment of persons with alveolar echinococcosis may reduce mortality (Schantz & Gottstein, 1986). By the time the disease becomes clinically manifest, the lesions have often progressed so that surgical complete resection is impossible. In a previous study we showed that a purified antigenic polypeptide (antigen Em2) from *E. multilocularis* demonstrated immunodiagnostic characteristics suitable to detect by ELSA early cases of alveolar echinococcosis among large groups of persons living in endemic areas (Gottstein, Schantz & Wilson, 1985; Gottstein *et al.* 1987). This was attributed to the operating characteristics (diagnostic sensitivity 94 %; specificity 100 % for non-*Echinococcus* reactions, 94 % with respect to *Echinococcus granulosus* infections) defining the Em2-ELISA (Gottstein, 1985); the test furthermore allowed a reliable discrimination of human cases of alveolar form cystic echinococcosis (Gottstein, Eckert & Fey, 1983; Gottstein *et al.* 1986). Field-application of the Em2-ELISA in the frame of sero-epidemiological screening of human populations resulted in the first published finding of spontaneously 'died-out' or 'aborted' *E. multilocularis* lesions in human patients (Rausch *et al.* 1987). These patients maintained a high anti-Em2 antibody concentration, despite the fact that only non-viable calcified parasite residues remained as lesions in the liver. Interestingly, anti-Em2 antibody concentrations dropped to negative rapidly after surgical resection of the liver lesion in question. The Em2-ELISA is now being established as a WHO reference immunodiagnostic test for alveolar echinococcosis (WHO/CDS/VPH/88·78). The Em2-ELISA was recently also investigated for assessing adult-stage *E. multilocularis* infections in definitive hosts (foxes), resulting in the hypothesis that the presence of anti-Em2 immunoglobulins rather reflected a limited post-oncospheral parasite development in the definitive host than the actual presence of intestinal *E. multilocularis* worms, thus indicating a metacestode-stage specificity of the antigen (Gottstein *et al.* 1991).

The present report describes the preparation of a species-specific anti-Em2 (*E. multilocularis*) monoclonal antibody for (i) further biological character-

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This publication is dedicated to Professor J. Eckert on the occasion of his 60th birthday.

ization of the Em2 antigen, (ii) easy purification of Em2 antigen for immunodiagnostic and immunological investigations and (iii) development and application of a sandwich-ELISA for the specific detection of Em2 antigen (and thus *E. multilocularis*) in diagnostic samples.

MATERIALS AND METHODS

Experimental design

Species-specific monoclonal antibodies (MAb) were generated against *Echinococcus multilocularis* with the following strategy. (i) Primary screening with ELISA of MAb with antigens reflecting species-specificity and genus-specificity, including cross-reactivity and non-specificity. Primary selection of MAb was based on the specific reaction with Em2 antigen (Gottstein, 1985). (ii) Characterization of Em2-binding MAb with the following techniques and aims. Sandwich-ELISA using solid-phase MAb and a dot-ELISA were performed using different kinds of parasite and control antigens. Both assays demonstrated Em2 specificity in relation to stage- and species-specificity. Further demonstration of stage-specificity was by direct immunofluorescence with native *E. multilocularis* parasite material of different stages and FITC-labelled MAb. (iii) A direct comparative analysis was made by ELISA of Em2-antigen purified with solid-phase MAb (antigen Em2(G11)) versus Em2 antigen (Gottstein, 1985), a purified recombinant II/3–10 antigen known to be not related immunologically to Em2 antigen (Müller *et al.* 1989) and a crude *E. multilocularis* somatic antigen. The investigation was done with characteristic and defined sera from human patients with alveolar echinococcosis.

Antigens

Metacestode tissue of a Swiss *Echinococcus multilocularis* isolate (CH24) maintained in jirds (*Meriones unguiculatus*) by intraperitoneal transplantation (Eckert & Pohlenz, 1976) was homogenized in a Polytron PCU-2 blender and frozen/thawed 3 times using liquid nitrogen and a +37 °C water-bath. Subsequently the material was ultrasonicated (60 s, 40 W, 80% pulse) and sedimented at 10000 ***g*** for 30 min at +4 °C. The supernatant fraction containing somatic antigens was used to immunize mice for monoclonal antibody production. An equivalently processed antigen but originating from a transplant in cotton rats (*Sigmodon hispidus*) was used for a first step MAb screening and pre-selection in ELISA. Purification of the species-specific *E. multilocularis* Em2 antigen was done exactly as described previously (Gottstein, 1985), this Em2 antigen was used in parallel for primary MAb screening. Somatic antigens were derived from the following *Echinococcus* materials for further serological characterization of MAb (using ELISA, dot-ELISA and sandwich-ELISA): (i) *E. multilocularis* metacestodes from other isolates than that listed above (number of isolates in parentheses) originating from Switzerland (8), France (3), Alaska (1), Germany (1) and Japan (2); (ii) gravid adult stage *E. multilocularis* tapeworms recovered from the mucosa of the small intestine from a naturally infected Swiss fox, washed 3 times with PBS prior to further processing; (iii) *E. granulosus* protoscoleces from hydatid cysts dissected from the lungs of naturally infected Swiss cattle; (iv) *E. vogeli* metacestodes from experimentally infected jirds (gift from Dr R. L. Rausch, University of Washington, Seattle). All *Echinococcus* somatic antigens were prepared as described in the paragraph above. *E. granulosus* hydatid cyst fluid was collected from fertile lung or liver cysts of the following host species (origin, number of isolates): Cattle (Switzerland, 13); horse (Switzerland, 2); sheep (Sardinia, 1); pig (Poland, 2); camel (Egypt, 1); human (Switzerland, 5). Non-*Echinococcus* somatic antigens were of following origin (origin, number of isolates): *Taenia solium* cysticerci (Mexico, 1; South Africa, 1); *Cysticerus bovis* (*T. saginata*) (Switzerland, 2); *T. crassiceps* metacestode (from experimentally infected BALB/c mice, 1); *Cysticercus tenuicollis* (*T. hydatigena*) (Switzerland, 1); *Mesocestoides corti* metacestode (from experimentally infected BALB/c mice, 1); mature adult stages from *Toxocara canis*, *Fasciola hepatica*, *Dicrocoelium dendriticum*; control tissues from muscles and liver of human, murine and bovine origin. All these additional somatic antigens were prepared exactly as described above. For obtaining excretory/secretory antigens (E/S-Ag) from *E. multilocularis*, a metacestode (CH 24 isolate) cell suspension (obtained by homogenization of metacestode tissue in a Tenbroek's homogenizer) was cultivated for 10 days in DMEM (Gibco) with 100 U penicillin G and 100 μg streptomycin per ml without foetal calf serum. Collected culture medium was dialysed and concentrated using an Amicon ultrafiltration unit and a YM-10 membrane. Purification of the recombinant *E. multilocularis* II/3–10 antigen was exactly as described by Müller *et al.* (1989).

All protein concentrations were assessed by the Bio-Rad protein assay and with bovine albumin as a standard.

Hatching of eggs and activation of oncospheres for in vitro *cultivation*

E. multilocularis eggs were isolated under bio-hazard conditions from adult-stage tapeworms recovered from the small intestine of naturally infected necropsied foxes. Basically the same procedure as described by Deplazes & Eckert (1988) for *T. hydatigena* was employed for isolation of viable *E. multilocularis* eggs

and subsequent oncosphere activation and *in vitro* cultivation. Embryophore disruption was done in 1 % sodium hypochlorite solution (1 % active chlorine concentration, pH 11) for 5 min at room temperature. Oncospheres were washed twice in PBS and once in 0·025 M HCl (in physiological saline solution) by sedimentation at 600 ***g*** for 10 min. Activation of the oncospheres was performed with a solution of 1 % pancreatin (Fluka), 15 % dog bile and 1 % $NaHCO_3$ in distilled water (sterilized by 0·25 μm pore size filtration) for 15–30 min at +37 °C with vigorous shaking once every 5 min. Activating solution was removed by washing with DMEM (Flow). Activated oncospheres were cultivated in 30 ml plastic tissue-culture flasks (Falcon) containing a monolayer of Swiss mouse embryo (3T3) cells (Flow) in 10 ml of tissue-culture medium (DMEM with 100 U penicillin G and 100 μg streptomycin (KC Biological) per ml with 10 % foetal calf serum). The culture medium was replaced on days 5, 9 and 13 of cultivation. The whole process was visually monitored in a Leitz Labovert FS microscope.

Generation of monoclonal antibody (MAb)

Female BALB/c mice aged 6 weeks were immunized by s.c. injection of 50 μm of somatic metacestode antigen of *E. multilocularis* (prepared as described above) emulsified in complete Freund's adjuvant (Difco). Three weeks later the procedure was repeated but with incomplete Freund's adjuvant. Ten days after the first boost the same amount of antigen diluted in 200 μl of PBS was injected intraperitoneally. Three to four days after this last boost, spleen cells of mice were fused with AG8 × 63 myeloma cells (provided by Dr A. Metzler, Institute of Virology, University of Zürich) using a 50 % polyethylene glycol (PEG) 1500 (Bioproducts) solution in serum free DMEM (Gibco). Fusion and cell-culture procedures were carried out as described by De St. Groth & Scheidegger (1980).

Ten to 15 days after fusion the supernatant fractions from wells containing hybridomas were screened by ELISA with somatic *E. multilocularis* metacestode antigen (cotton rat isolate) and Em2 antigen. Supernatants positive in primary screening were subsequently tested in ELISA with somatic *E. granulosus* protoscoleces, *T. hydatigena* and *T. solium* metacestode antigen. Hybridomas selected for species-specific anti-Em2 antibody synthesis (and others) were recloned 3 times by limiting dilution and preamplified in 30 ml plastic tissue-culture flasks (Falcon) for generation of ascites-producing tumours in BALB/c mice. Pristane (1 ml/animal) was injected i.p. into the animals 15 days and 3 days prior to i.p. injection of 10×10^6 hybridoma cells. Ascites fluid was harvested after necropsy 2–3 weeks later and stored frozen at −20 °C.

Purification and isotyping of MAb

Anti-Em2 (and other) MAb were partially purified from ascites by precipitation with 50 % saturated ammonium sulphate (Fleischmann, Pain & Porter, 1962).

MAb isotypes were determined by immunodiffusion ('mouse monoclonal typing kit', The Binding Site Ltd).

ELISA for MAb

The ELISA used for screening and selection of MAb (from supernatants of hybridoma cell-cultures) was carried out as previously described (Gottstein, Eckert & Fey, 1983) with the following modifications. The polystyrene surface of MicroELISA plates (Nunc-Immuno Plate MaxiSorp, No. 4-39454A) was coated with antigen (5 μg protein antigen/ml) in 100 μl of 0·1 M $NaHCO_3/Na_2CO_3$, pH 9·6 (+0·02 % NaN_3) at 4 °C overnight in a humid chamber. The wells were then washed 3 times with PBS + 0·3 % Tween 20 (PBS/Tween) and incubated with the same buffer for 20 min. Supernatants were added undiluted to each well and incubated for 2 h at 37 °C. After 3 washes (PBS/Tween) 100 μl of sheep anti-mouse IgG-IgM-IgA-alkaline phosphatase conjugate (The Binding Site Ltd) was incubated in a 1 : 500 dilution (PBS/Tween) for 2 h at 37 °C. Further procedures for visualization of the serological reaction corresponded to those described previously (Gottstein *et al.* 1983).

Antigen detection by sandwich-ELISA

The polystyrene surface of MicroELISA plates was coated with 5 μg (/well) of partially purified anti-Em2 MAb G11 and control MAb G10/4 (*Giardia lamblia*-specific (Aggarwal, Merritt & Nash, 1989), generously provided by Dr T. E. Nash, LPD, NIAID, NIH, Bethesda) as described above. Somatic- and E/S-antigens of various helminth species and control tissues were examined for the presence of epitopes binding uniquely to MAb G11 at a concentration of 50 μg/ml PBS-Tween. MAb G11 coupled to alkaline phosphatase from calf intestine (Grade I, Boehringer Ltd) according to a standard method (Engvall & Perlmann, 1972), was used as conjugate (1 : 200 dilution in PBS-Tween for 1 h at room temperature) for detecting primary antibody–antigen reactions. The conjugate was stored as a 50 % glycerol dilution at −20 °C.

Dot-ELISA

Soluble antigen preparations were spotted as 3 μl drops (0·5 mg protein/ml PBS) onto strips of nitrocellulose (BA 85, Schleicher & Schuell). Strips

were air-dried at room temperature for 2 h, washed 3 times with PBS-Tween, the last wash solution was left for 30 min. Strips were then incubated with alkaline phosphatase-conjugated MAb G11 (1:500 in PBS–Tween) for 2 h at room temperature. Visualization of antigen–MAb G11 (alkaline phosphatase-labelled) complexes was performed as described by Dao (1985).

Direct immunofluorescence assay

The presence of antigens reactive to MAb was also determined using direct immunofluorescence. For that purpose anti-*E. multilocularis* MAb G11 and control MAb G10/4 were both conjugated to fluorescein isothiocyanate according to standard procedures. Freshly collected (from naturally or experimentally infected animals) adult-stage *E. multilocularis* tapeworms, *E. multilocularis* metacestodes (protoscoleces and germinal layer including laminated layer) and *E. multilocularis* oncospheres after hatching and *E. multilocularis* oncospheres after 13 days of *in vitro* cultivation were all washed 3 times in PBS prior to incubation (at 4 °C) with FITC-labelled MAb G11 or control MAb G10/4 (1:20 final dilutions) for 30 min. After repeated washes (3 times with PBS) the fluorescence was monitored with an Olympus BH-2 microscope and photographed onto Kodak Ektachrome EPY-50 film.

Affinity purification of antigens

Partially purified ascites (MAb G11 and control MAb G10/4) was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia Fine Chemicals). Affinity chromatography was performed as described previously by Baumann & Gottstein (1987). Briefly, somatic metacestode antigen (0·5 mg) from *E. multilocularis* (CH10 isolate) was incubated with the antibody–Sepharose (5 ml of slurry, gel previously equilibrated with PBS containing 0·5 M NaCl) for 2 h at room temperature by slowly rotating the chromatography column. After washing the gel thoroughly with PBS (0·5 M NaCl) the bound protein was eluted with 5 M $MgCl_2$. The eluate was dialysed against PBS and concentrated by ultrafiltration as described above, storage was at −80 °C until use. The eluate obtained from solid-phase G11 MAb was designated Em2(G11).

Sera

The sera used for analytical and comparative investigations of different *E. multilocularis* antigens were obtained from 30 patients with clinically, parasitologically or histologically proven alveolar echinococcosis (*Echinococcus multilocularis*). The group was statistically matched by sex and age. All sera were subjectively pre-selected from previous studies according to their known and quantitatively varying reactivity with Em2-antigen. The data resulting from the present study thus do not reflect operating characteristics of antigens such as diagnostic sensitivity.

ELISA for human serum antibodies

All sera listed above were simultaneously tested in parallel runs with ELISA using *E. multilocularis* somatic antigen (CH10 isolate), Em2 antigen (Gottstein, 1985), Em2(G11) and recombinant *E. multilocularis* II/3-10 antigen (Müller *et al.* 1989). The latter antigen was included as a control *E. multilocularis* antigen not related to Em2. The ELISA technique employed corresponded to that described previously (Müller *et al.* 1989).

RESULTS

Generation of hybridomas and selection of MAb

Five fusions were performed. Supernatants (cell culture medium taken from growing hybridoma cells) were tested in ELISA with somatic antigen from *E. multilocularis* metacestodes (cotton rat isolate). All in all, 26 supernatants were considered antibody-positive ($A_{405\,nm}$ at least >10 times the negative control value). Subsequently, positive supernatants were tested in ELISA with Em2 antigen (Gottstein, 1985), and also with *E. granulosus* hydatid cyst fluid (bovine isolate) and somatic antigens derived from *T. hydatigena* and *T. saginata* metacestodes. One single cell line out of the 26 positive showed a strong reaction with all antigens investigated and thus was disposed as a non-specific MAb-producing cell line. All other 25 cell lines showed strong reactivity to Em2 antigen in ELISA, and 10 from them were also positive with *E. granulosus* hydatid cyst fluid antigen but negative with *T. hydatigena* and *T. saginata* metacestode antigen.

One MAb (G11, IgG_1 isotype) which reacted uniquely with crude *E. multilocularis* metacestodes and purified Em2-antigen (therefore assumed to be *E. multilocularis* species-specific) was selected for further cloning and production of ascites fluid.

MAb G11 binding properties in sandwich-ELISA and dot-ELISA

A solid-phase MAb G11 sandwich-ELISA was used as a first step to establish the operating characteristics such as specificity (Fig. 1) and methodical sensitivity (Fig. 2).

Specificity of the assay was tested by investigating 15 isolates of *E. multilocularis* versus 24 isolates of *E. granulosus*, 1 isolate of *E. vogeli* and various non-

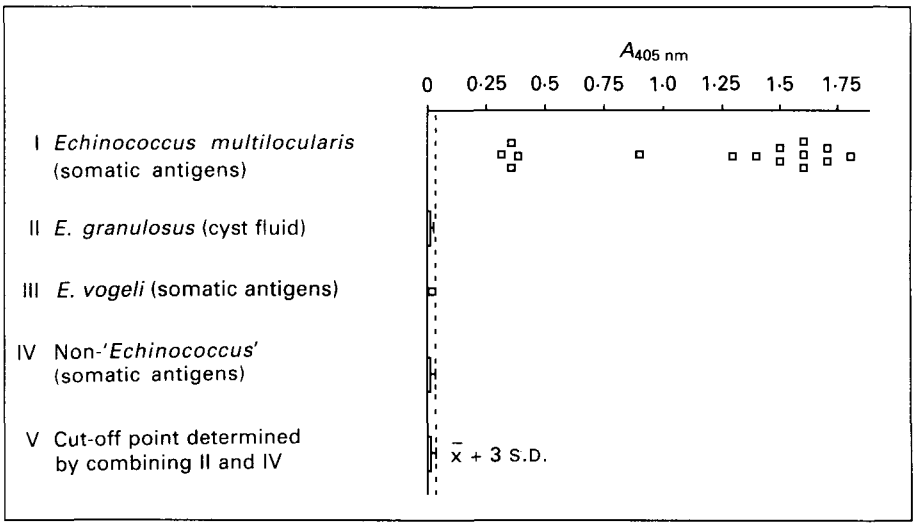

Fig. 1. Specificity of MAb G11 sandwich-ELISA with antigens tested at a concentration of 50 μg protein/ml. *I*, *Echinococcus multilocularis*, 15 isolates originating from Switzerland, France, Alaska, Germany, Japan and Canada. *II*, *E. granulosus* hydatid cyst fluids from bovine (n = 13), equine (n = 2), porcine (n = 2), ovine (n = 1), camel (n = 1) and human (n = 5) origin. *III*, *E. vogeli* somatic metacestode antigen. *IV*, Non-*Echinococcus* somatic antigens were from *Taenia solium* cysticerci (n = 2), *T. saginata* cysticerci (n = 3), *T. crassiceps* metacestodes (n = 1), *T. hydatigena* metacestodes (n = 2), *Mesocestoides corti* metacestodes (n = 1). Adult-stage somatic antigens were from *Toxocara canis*, *Fasciola hepatica* and *Dicrocoelium dendriticum*, control extracts from muscle and liver of human, mice and cattle origin. *V*, The cut-off threshold determining the lower resolving limit was calculated on the basis of $A_{405\ nm}$ values from *II* and *IV*.

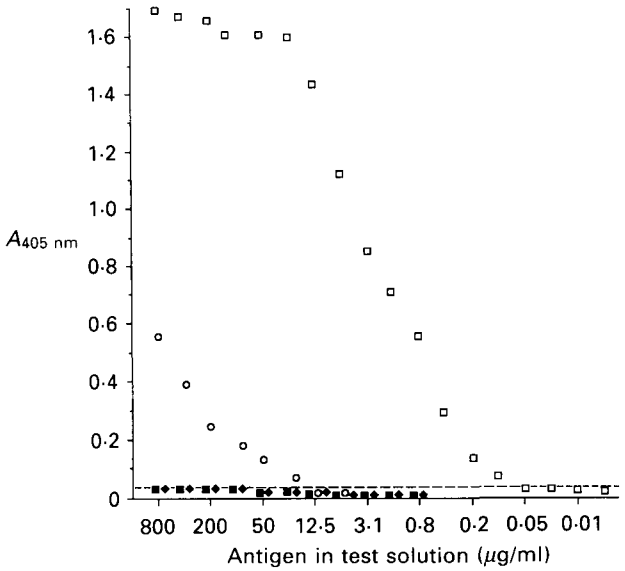

Fig. 2. Titration of (□) *Echinococcus multilocularis* somatic antigen, (◆) *E. granulosus* hydatid cyst fluid antigen and (■) *Taenia solium* cysticercus somatic antigen by MAb G11 sandwich-ELISA. *E. multilocularis* somatic antigen was also controlled by irrelevant MAb G10/4 sandwich-ELISA (○). The lower resolving limit corresponds to that shown in Fig. 1.

Echinococcus helminth and control antigens (listed in detail in Fig. 1). The assay was controlled by simultaneous testing on solid-phase irrelevant G10/4 MAb. The respective control values (never exceeding 0·02 $A_{405\ nm}$ for all non-*E. multilocularis* antigens and 0·20 $A_{405\ nm}$ for all *E. multilocularis* antigens) were individually subtracted from the value obtained with the specific solid-phase MAb G11. The data show that all *E. multilocularis* isolates were clearly detected by the present sandwich-ELISA, and specificity was 100% due to absolutely no binding activity with any antigens derived from other *Echinococcus* species, other helminths or control tissues. All antigen concentrations had been equivalently adjusted to 50 μg protein/ml after optimizing test parameters and determining methodical sensitivity (see Fig. 2). Arbitrarily, we selected a positive/negative threshold characterized by a cut-off $A_{405\ nm}$-value set at $\bar{x}$+3S.D. from all non-*E. multilocularis* isolates. The methodical sensitivity was determined by plotting titration values obtained with primary solid-phase MAb G11 and values from solid-phase irrelevant control MAb G10/4, including sequentially homologous *E. multilocularis* metacestode antigen and MAb G11-alkaline phosphatase conjugate in this reaction (Fig. 2). Based on this experiment the final methodical sensitivity was depicted as a concentration of 80 ng *E. multilocularis* protein/ml test solution. For demonstration of insignificant background-reactivity the figure includes also *E. granulosus* and *T. solium* antigens tested on solid-phase MAb G11, data of the corresponding control reactions with G10/4 are not shown as values never exceeded 0·02 $A_{405\ nm}$. Testing titrated *E. multilocularis* antigen with solid-phase control MAb G10/4 showed some non-specific reaction at concentrations larger than 13 μg protein/ml. These

Methods / Antigens	Dot-ELISA	Sandwich ELISA Control MAb G11	Sandwich ELISA Control MAb G10/4
I *Echinococcus multilocularis*		$A_{405\ nm}$	
Metacestode somatic antigen	●	0·887	0·117
Metacestode E/S-antigen	●	0·811	0·023
Adult (gravid) somatic antigen		0·002	0·004
Oncosphere somatic antigen		0·002	0·004
Oncosphere (cultivated 13 days *in vitro*) somatic antigen	●	0·710	0·050
II Purified *E. multilocularis* antigens			
Em2	●	0·785	0·051
Em2(G11)	●	0·516	0·008
Em II/3-10		0·002	0·002
III Control antigens			
Mouse liver somatic antigen		0·002	0·007
Mouse serum		0·006	0·006
Calf serum		0·001	0·002
Culture medium		0·004	0·016
E. granulosus protoscolex somatic antigen		0·002	0·006

Fig. 3. Specificity of dot-ELISA and sandwich-MAb G11- and control MAb G10/4-ELISA.

values, reflecting minor non-specificity of *E. multilocularis* antigen components (they were never observed with any other antigens tested), were subsequently corrected by subtraction, similar to the corrections performed in Fig. 1.

Analysis of stage-specificity by sandwich-ELISA and dot-ELISA (Fig. 3 *I*) revealed that only antigens derived from the metacestode provided epitodes with MAb G11-binding properties. No reactivity of MAb G11 was observed with antigens from adult-stage tapeworms and with oncospheres freshly hatched from *E. multilocularis* eggs. MAb G11, however, was able to react with antigen derived from oncospheres cultivated *in vitro* for 13 days. For further analytical characterization of the antigen with MAb G11-binding epitope(s), the previously purified Em2 antigen (Gottstein, 1985) and the recombinant II/3-10-antigen (Müller *et al.* 1989), known to be not related to Em2, were included in the investigation. The respective results shown in Fig. 3 *II* confirm the Em2 specificity of MAb G11 or, in other words, proving that the Em2 antigen carries the MAb G11-binding epitope. As expected, the recombinant II/3-10 antigen, as a negative control, exhibited no binding activity to MAb G11. In parallel to the sandwich-ELISA, the corresponding immunological reactions were tested by dot-ELISA with the same antigens but in solid-phase. Serological results were qualitatively identical to those obtained in sandwich-ELISA.

All control antigens (Fig. 3 *III*) showed no binding properties in the MAb G11- and control MAb G10/4-sandwich-ELISA.

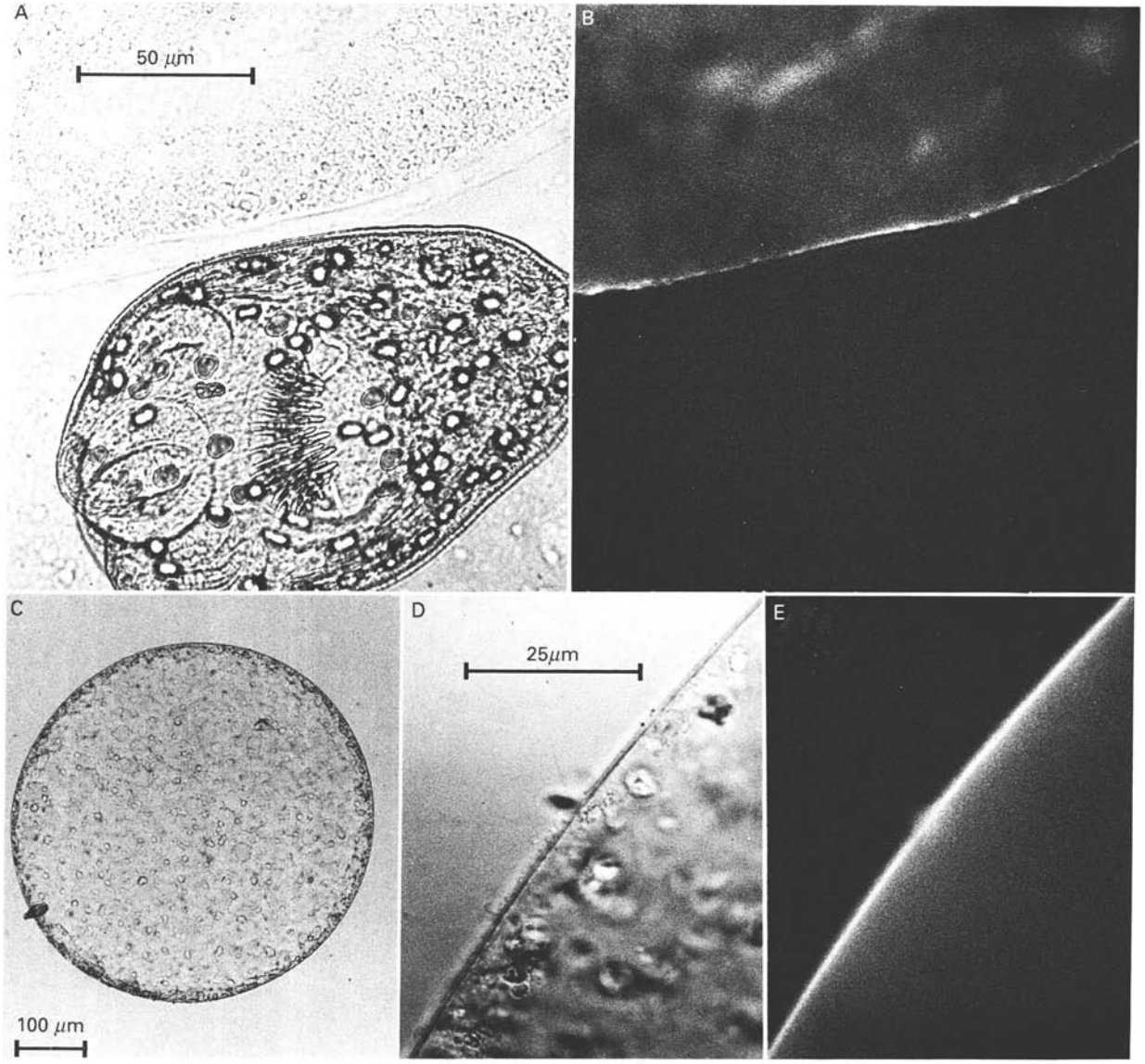

Fig. 4. Direct immunofluorescence analysis of the localization of the MAb G11-binding Em2 antigen. (A) MAb G11-FITC-labelled native *Echinococcus multilocularis* tissue under normal light. (B) The same as in (A) but under fluorescence. (C and D) MAb G11-FITC-labelled native oncosphere, *in vitro* cultivated for 13 days, under normal light. (D) Magnified section of (C). (E) The same as (D) but under fluorescence.

Characterization of MAb G11 binding properties by fluorescence microscopy

Fluorescence microscopy analysis showed that from native *E. multilocularis* metacestode tissues only the laminated layer was stained by MAb G11, with a relatively strong surface fluorescence (Fig. 4). Native protoscoleces did not appear to bind MAb G11, nor did adult-stage *E. multilocularis* tapeworms or oncospheres freshly hatched from *E. multilocularis* eggs (data for the latter two not shown). Oncospheres cultivated *in vitro* developed into spherical organisms with a surrounding thin layer. This layer demonstrated specific binding of MAb G11 at day 13 of *in vitro* cultivation.

Control reactions performed with irrelevant MAb G10/4 revealed that all parasite tissues investigated did not non-specifically bind irrelevant mouse IgG.

Overall, the fluorescence microscopy analysis was in perfect agreement with the results from sandwich- and dot-ELISA.

ELISA with Em2(G11)-antigen

In order to determine the immunodiagnostic characteristics of the affinity-purified Em2(G11) antigen, an analysis was performed by ELISA in comparison with other *E. multilocularis* antigens listed below and in Fig. 5. The results of investigating sera from human patients with alveolar echinococcosis clearly revealed similar operating characteristics for Em2(G11) and Em2 (Gottstein, 1985) antigens, demonstrated by the relatively high

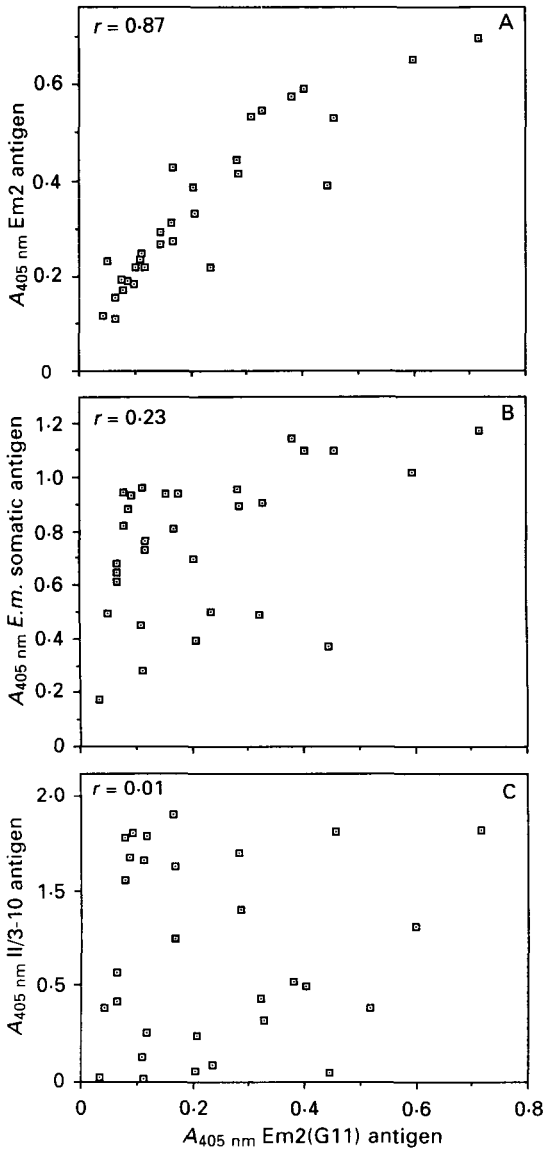

Fig. 5. Direct comparative analysis by ELISA of Em2 antigen purified with solid-phase MAb (antigen Em2(G11)) versus (A) Em2 antigen (Gottstein, 1985), (B) a crude *Echinococcus multilocularis* somatic antigen and (C) a purified recombinant II/3-10 antigen known to be unrelated immunologically to Em2 antigen (Müller *et al.* 1989); the investigation was done with defined sera from 30 human patients with alveolar echinococcosis (see Material and Methods section).

correlation coefficient ($r = 0{\cdot}87$). Crude *E. multilocularis* somatic antigen (CH10 isolate) also exhibited some minor degree of correlation with the Em2(G11) antigen ($r = 0{\cdot}23$), whereas the recombinant II/3-10 antigen (Müller *et al.* 1989) demonstrated no statistical correlation with the Em2(G11) antigen ($r = 0{\cdot}01$).

DISCUSSION

In this paper we describe a murine monoclonal antibody (designated MAb G11) that specifically reacts with an epitope present on the previously characterized Em2-antigen of *E. multilocularis* metacestodes (Gottstein, 1985). The reactivity of this antibody was investigated by sandwich-ELISA and dot-ELISA with various isolates of *E. multilocularis* metacestodes for demonstrating that the epitope corresponding to MAb G11 was ubiquitously presented by all isolates. This observation confirmed directly the demonstration of conservation of the Em2 antigen previously assessed by indirect anti-Em2 antibody detection in human patients with alveolar echinococcosis, originating from geographically dispersed endemic areas (Gottstein *et al.* 1986). Direct proof for the localization of the epitope recognized by MAb G11 on the Em2 antigen was found by investigating affinity-purified (according to Gottstein (1985)) Em2 antigen in sandwich- and dot-ELISA. In both test systems, MAb G11 showed binding activity to the Em2 antigen, whereas a negative control antigen (recombinant II/3-10 antigen, known to be not related to the Em2 antigen) remained negative as expected. Indirect evidence for Em2 identity was also obtained by a comparative investigation of sera from human patients with alveolar echinococcosis in ELISA with different solid-phase antigens: Em2(G11) versus Em2 (Gottstein, 1985) demonstrated statistically a good correlation. Some degree of correlation occurred also with somatic *E. multilocularis* metacestode antigen. This was to be expected because of the relatively high proportion of Em2 antigen observed previously in somatic extracts (Gottstein *et al.* 1983; Gottstein, 1985).

Of considerable interest was the observed metacestode stage-specificity of the *E. multilocularis* epitope binding MAb G11 and being localized on the Em2 antigen. According to direct immunofluorescence analysis, the molecule with MAb G11-binding activity appeared to be accumulated in the laminated layer either adjacent to the germinal layer or to oncospheres developed *in vitro* for 13 days. The laminated layer is known to remain for extremely long times in infected host tissue, even after spontaneous 'dying-out' of the larval parasite (Rausch *et al.* 1987). The remarkably long persistence of anti-Em2 antibodies in serum from patients with such aborted lesions hence becomes explained, as well as the very rapid decrease of anti-Em2 antibody concentrations (finally becoming negative) observed after complete surgical resection of such dead lesions (Lanier *et al.* 1987; Gottstein *et al.* 1989).

Structures of the native germinal layer did, in general, not bind MAb G11 in the direct immunofluorescence analysis, although some individual, not further defined cells appeared fluorescent (data not shown). The acellular nature of the MAb G11-positive laminated layer still requires clarification of (i) the localization of the site of production of the epitope in question, (ii) the nature of the respective

synthesizing parasite cells and (iii) the exact biochemical properties of the molecule(s) carrying the MAb G11-binding epitope. Such experiments are presently under investigation.

In conclusion, the development of a species-specific monoclonal antibody against an epitope of the Em2 antigen proved useful for further biological characterization and purification of the Em2 antigen. Furthermore, the application of MAb G11 in a sandwich-ELISA demonstrated also its potential value for the diagnostic identification of *E. multilocularis* metacestodes and simultaneously for its discrimination from various other cestode, helminthic or bacterial cell extracts.

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